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Nitrate Metabolism in Plants under Hypoxic and Anoxic Conditions

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ABSTRACT

Plants are frequently exposed to a variety of external conditions able to affect their growth, development and productivity. Their ability to adapt and live in a changing environment relies on tolerance or resistance to adverse growing seasons. Oxygen limitation is the major abiotic stress in flooded soils. In the open field, during winter or the raining season in poorly drained soils, plants can experience waterlogging, an event causing scarce root oxygenation and leading to a hypoxic stress. The metabolism of plants undergoes deep modifications in order to minimize energy losses, the most important changes concerning of course glycolysis. With the reduction of oxygen availability specific anaerobic enzymes become activated and their transcripts are over-expressed. Fermentation is induced and potentially toxic metabolites like ethanol are accumulated into anoxic tissues. Among the metabolic pathways strongly affected by oxygen deficiency stands also nitrate metabolism. The enzymes involved in nitrate assimilation and reduction are up-regulated during oxygen deficiency and some authors showed several correlations between nitrate availability and plant survival to flooding. Moreover nitrites and nitric oxide seem to be directly involved in plant survival at least during the first hours after the onset of hypoxia/anoxia. Intracellular nitrates and nitrites concentrations may play an effective role in maintaining cellular pH homeostasis, limiting cytoplasmic acidosis deriving from fermentation and thus contributing to survival. This mini-review is intended to summarize and discuss nitrate metabolism under limited oxygen conditions with a brief introduction on the metabolic pathways that interact with nitrate assimilation.

Keywords: anaerobiosis, anoxia, enzymes, fermentation, genes, nitrite

Abbreviations: ADH, alcohol dehydrogenase; **HATS**, *h*igh *a*ffinity *t*ransporter *s*ystem; **LATS**, *l*ow *a*ffinity *t*ransporter *s*ystem; **LDH**, lactate dehydrogenase; **NR**, nitrate reductase; **NiR**, nitrite reductase; **NFT**, Nutrient Film Technique; **PDC**, pyruvate decarboxylase

CONTENTS

INTRODUCTION 136
  Greenhouses and oxygen deficiency 137
  Phenotypic and metabolic changes in response to anaerobiosis 137
METABOLIC CHANGES IN PLANTS UNDER LIMITED OXYGEN CONDITIONS: FERMENTATION AND PH 137
NITRATE METABOLISM IN PLANTS UNDER HYPOXIA OR ANOXIA 138
  Nitrate uptake and assimilation in normoxic conditions 138
  Limited oxygen conditions and nitrate metabolism 138
    NR regulation, nitrate and nitrite 138
    Aa accumulation 140
    NO and haemoglobin 140
CONCLUSIONS 140
REFERENCES 140

INTRODUCTION

Plants can modify their genetic expression pattern, intracellular metabolism and growing ability in order to suit the changed environment. Thanks to a high plasticity plants can survive in extreme environments and overcome the difficult periods of the year. Constantly many biochemical pathways are being activated and many others are being repressed (Tennis *et al*. 2000) to achieve the task. Usually a specific stress is associated to the activation of a specific gene that may work as a signal for cascade activation events (Drew 1997). The relationship between plants and stresses may cover all aspects of plant physiology.

Anaerobiosis is a universal biological phenomenon experienced to some extent by all organisms and for plants this is quite a common event. During winter or by seasonal flooding plants may suffer from waterlogging, but water-saturated soils can also be a consequence of excessive irrigation or of a soil with poor drainage. In these conditions, plants become stressed by low radical oxygen availability which causes a drop in tissues O_2 supply and leads to hypoxia or even anoxia. In agriculture, both hypoxia and anoxia may reduce crop productions. In fact, crop yields are dramatically compromised after a long period of waterlogging: injuries depend on plants ability to bear hypoxic conditions, ranging from submerging sensitivity to submerging tolerance and this trait is species-dependent. In general the survival to oxygen deprivation depends on which plant tissue type is involved, the developmental stage, the genotype, the severity and duration of the stress, light levels and temperature (Fukao and Bailey-Serres 2004).

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Invited Mini-Review

Greenhouses and oxygen deficiency

Limited oxygen availability may naturally occur in flooded lands, but it can also be a problem in hydroponic growing systems of modern greenhouse cultivations. Nutrient Film Techniques (NFT) and floating systems are soilless cultivation systems that do not have substrates and roots are directly immersed into a nutrient solution. Water and nutrients are not discharged but collected and reused with depletion of mineral elements and oxygen. During summer, especially for cultures with long growing cycles, oxygen depletion may generate hypoxia to anoxia with risks of plant injuries and loss in productivity (Pardossi *et al.* 2005). Roots are usually the main organs to suffer from oxygen deficiency as the aerial part of the plant remains aerated beyond water. However, the effects of a radical oxygen shortage can be sensed by the shoot as well and it can affect the whole plant metabolism, depending on the exposure time.

Phenotypic and metabolic changes in response to anaerobiosis

According to Drew (1997), *normoxia* is defined as the condition in which oxygen supply does not limit oxidative phosphorylation. During *hypoxia* oxygen partial pressure is low enough to limit the production of ATP by mitochondria, whereas *anoxia* is attained when the mitochondrial production of ATP is insignificant compared to that generated by glycolysis and fermentation.

Thus, the ability of plants to survive under complete submergence even for weeks comes from their energy supply (Perata and Alpi 1993). Some plant species are able to grow and reach the reproductive stage depending on the strategies they adopt to produce ATPs and maintain the glycolitic flux. Plants can modify their morphology and tissue anatomy – aerenchyma formation and epinastic growth are examples – only when long periods of oxygen shortage occur, but they immediately respond to oxygen drops by modulating and activating specific metabolic pathways (Drew 1997; Perata and Voesenek 2007).

Oxygen deficiency is first sensed by mitochondria as cytochrome oxidase activity becomes oxygen limited, due to the lack of terminal electron acceptors for the electron transport chain. Hypoxic mitochondria undergo a structural remodelling possibly leading to swelling and destruction (Vartapetian *et al.* 2006), but as a first response to oxygen drops they release Ca^{2+} ions in the cytoplasm (Subbaiah and Sachs 2003). Ca^{2+} is believed to function as a signal molecule for anaerobiosis and in maize seedlings has been shown to enter the nucleus possibly to activate the transcription of genes involved in plant stress response (Subbaiah *et al.* 1994). A recent work on the hypoxic response in *A. thaliana* reported the induced expression of multiple genes encoding proteins whose activity is Ca^{2+}-regulated or that are capable of altering cytosolic Ca^{2+} concentrations (Liu *et al.* 2005).

Under oxygen deficiency, along with the alteration of gene transcriptional levels, plant metabolism switches from respiration to fermentation. The first genes to be upregulated encode for the so-called *ANPs* (anaerobiosis related proteins), which are mainly represented by enzymes involved in fermentative pathways (Sachs *et al.* 1980; Sachs *et al.* 1996; Huang *et al.* 2005).

With no oxygen availability oxidative respiration is blocked, the tricarboxylic acid cycle functions only partially and ATP has to be produced through less efficient pathways such as glycolysis, lactic and ethanolic fermentation, with the resulting accumulation of ethanol, lactate and Ala. As a consequence, intracellular pH values decline due to lactic acid production and proton accumulation, NADH levels increase while NAD^+ are rapidly consumed and ATP levels decrease. The ATP yield drops from 36 molecules produced by respiration per glucose to 2 ATP molecules produced by fermentation.

This state would lead to plant death unless some metabolic adaptation mechanisms delay the occurrence of severe injuries by buffering some toxic effects of fermentation.

Therefore the generalised biochemical basis for anoxia tolerance involves the maintenance of the redox and the energetic status of the cell through regulation of cytosolic pH, maintenance of glycolysis for ATP generation, regeneration of NAD^+ from NADH and formation of metabolic end-products that are innocuous or readily transported to the external solution. In the last decade, a growing attention has been paid to the positive effects that nitrate supply exerts on waterlogged plants and on anoxic tissues. Several evidences show that nitrate plays an important role in plant protection against anoxic damage and hypothesis have been proposed linking some branches of nitrate metabolism to hypoxic tolerance (Fan *et al.* 1988; Reggiani *et al.* 1985; Igamberdiev and Hill 2004). Some work has been done to try and dissect the biochemical and molecular mechanisms underlying the anaerobic nitrate metabolism and its correlation with cell energetic status and plant survival. In the following sections, changes of nitrate metabolism in plants under hypoxic and anoxic conditions are described and discussed.

METABOLIC CHANGES IN PLANTS UNDER LIMITED OXYGEN CONDITIONS: FERMENTATION AND PH

When oxygen concentration declines in the rhizosphere, metabolism shifts from aerobic to anaerobic and cytoplasmic pH in the root rapidly drops. During hypoxia and anoxia enzymes such as lactate dehydrogenase (LDH, E.C. 1.1.1.27), alcohol dehydrogenase (ADH, E.C. 1.1.1.1) and pyruvate decarboxylase (PDC, E.C. 1.2.4.1) are among the first to be activated. Nevertheless, LDH is a pH-sensitive enzyme unable to work under acidic conditions. It is rapidly upregulated upon O_2 deprivation but gets soon inhibited as lactic acid is produced, since this contributes to the decrease of the intracellular pH value. Thus during oxygen deficiency both PDC and ADH stay active and contribute to ethanolic fermentation, while lactic fermentation is a minor component of plant metabolism (Kennedy *et al.* 1992). Among the metabolites produced in anoxic tissues, ethanol is the most abundant. Assessment of ethanol distribution within the plant has showed that upon waterlogging, roots accumulate more ethanol than leaves. On the other hand laboratory experiments performed on wheat and rice with anaerobic chambers, in which oxygen deprivation was applied to whole plants or seedlings, showed that anaerobic metabolites are prevalent in shoots compared to roots (Mustroph *et al.* 2006). It has been proposed a beneficial role for ethanol on plant tolerance to anoxia as maize null ADH mutants survive only few hours of anoxia (Johnson *et al.* 1994) whereas tobacco, rice and Arabidopsis plants overexpressing the PDC gene accumulated a great amount of acetaldehyde and ethanol resulting in an improved tolerance to oxygen deficiency (Vartapetian 2006).

Cytoplasmic acidosis is the main cause of cell death during anoxia and is mainly caused by fermentation end-products. In addition, other sources of protons have been suggested to increase acidosis during anoxia. Libourel *et al.* (2006) reported a possible vacuolar H^+ leakage as they observed that cytoplasmatic pH drops rapidly and consistently while the vacuolar pH slightly decreases.

During anaerobiosis, cells try to keep the proton concentration under control by actively translocating them out of the cell membrane. But since H^+ pumps are ATP-dependent the cytoplasmatic ATP concentration strongly decreases. The reduced ATP availability, affects the activity of ATPase pumps leading to a complete inactivation. Prolonged anoxia induces the inactivation of all co-transporters that consumes ATP.

NITRATE METABOLISM IN PLANTS UNDER HYPOXIA OR ANOXIA

Although a drop in the pH value negatively affects radical nutrient uptake, mechanisms involving nitrate assimilation and reduction have been shown to be critical for plant survival to oxygen deficiency (Igamberdiev and Hill 2004).

In particular, a low pH value causes the reduction of nitrate assimilation, being its influx regulated by a proton-based symport. Nevertheless NO_3^- reduction exerts a beneficial effect on cytoplasmic pH acidosis and in maintaining higher ATP levels (Oberson *et al.* 1999). Until the mid of the '90s any positive effect observed and related to nitrate supply during oxygen deficiency had been explained by considering NO_3^- an alternative electron acceptor (Reggiani *et al.* 1993, Reggiani *et al.* 1985). According to this view, nitrate would be a substitute for oxygen as the final acceptor of the mitochondrial electron chain and its supply would sustain glycolysis. More recent studies demonstrated that the role of nitrate assimilation under anaerobiosis as a possible source of final acceptors is reductive, even though such a role has not been excluded. Thanks to new findings, nitrate is now considered as an intermediate metabolite in a cycle able to re-oxidize the reduced cofactors NADH, thus buffering the redox energy status of the anoxic cell (Igamberdiev and Hill 2004; Igamberdiev *et al.* 2005).

Nitrate uptake and assimilation in normoxic conditions

Nitrate movements through cellular membranes are always mediated by specific transporters. The translocation of NO_3 ions is active and involves both high and low affinity proton symporters (Crawford and Glass 1998). In roots, three distinct groups of nitrate transporters have been identified. Two of them have been classified as *h*igh *a*ffinity *t*ransporter *s*ystem (HATS) and include the *i*nducible (iHATS) and *c*onstitutive (cHATS) transport systems. Another group is represented by a constitutive *l*ow *a*ffinity *t*ransporter *s*ystem (LATS) (Kronzucker *et al.* 1995; Forde 2000; Thornton 2004). All these transporters are up-regulated by nitrate availability.

Nitrate influx into the root cells is proton coupled, as above mentioned, therefore nitrate transport is dependent on the H^+-ATPase pumps and it requires energy (Crawford and Glass 1998; Forde 2000; Forde 2002).

The assimilation of 1 mole of nitrate by roots generates almost 1 mol of OH^-. In leaves, instead, for one mole of nitrate, the OH^- produced may range from 0–1 mole, depending on plant species (Bolan *et al.* 1991). It has been proposed that in both symporter systems, for each NO_3^- loaded, two H^+ cross the plasma membrane and that this mechanism is tightly regulated by cellular pH (Ritchie 2006).

Plant nitrate metabolism is today well characterised and its key enzymes have been extensively studied.

Nitrate in plants can be reduced to nitrite both in roots and in shoots or loaded and stored inside the vacuole. NO_3^- reduction is catalysed by the enzyme Nitrate Reductase (NR, EC 1.6.6.1-3). NR in the leaf is a cytoplasmic enzyme and works in the presence of NAD(P)H, whereas in the root two distinct types of nitrate reductase are present: a cytoplasmic isoform (cNR) and a plasmamembrane-bound isoform (PM-NR), the latter also able to oxidize succinate (Stohr and Ullrich 1997). In normal conditions, NR reduces nitrates to nitrites, which are then transferred to the chloroplast/plastid where they are reduced to ammonium by nitrite reductase (NiR, EC 1.6.6.4). At last, ammonium is organicated to glutamate by glutamine synthetase (GS, EC 6.3.5.1) to produce glutamine (Stitt 1999). Therefore, nitrate is assimilated into amino acids via the GS-GOGAT pathway (GS1, GS2, GOGAT), resulting in glutamine and glutamate as primary N organic compounds (Tobin *et al.* 1985). Glutamine synthetase (GS) and glutamate synthase (GOGAT, EC 1.4.1.14) are the enzymes involved in ammonium assimilation, either derived from nitrate reduction, photorespiration or, in case it is externally supplied, as NH_3 and or NH_4^+ (**Fig. 1**). In roots, NO_2^- is converted into NH_4^+ mainly by the cytoplasmic GS isoform (named GS1), whereas the plastidial isoform (GS2) is less active. The opposite happens in the leaf, where the chloroplastic GS2 is the most active isoform.

NR activity is a key enzyme tightly regulated by many environmental and physiological factors (Wray 1988; Campbell 1999) and nitrate reduction represents the most important check-point in the metabolism of nitrous compounds.

Limited oxygen conditions and nitrate metabolism

NR regulation, nitrate and nitrite

Both anoxia and hypoxia are stress conditions able to in-

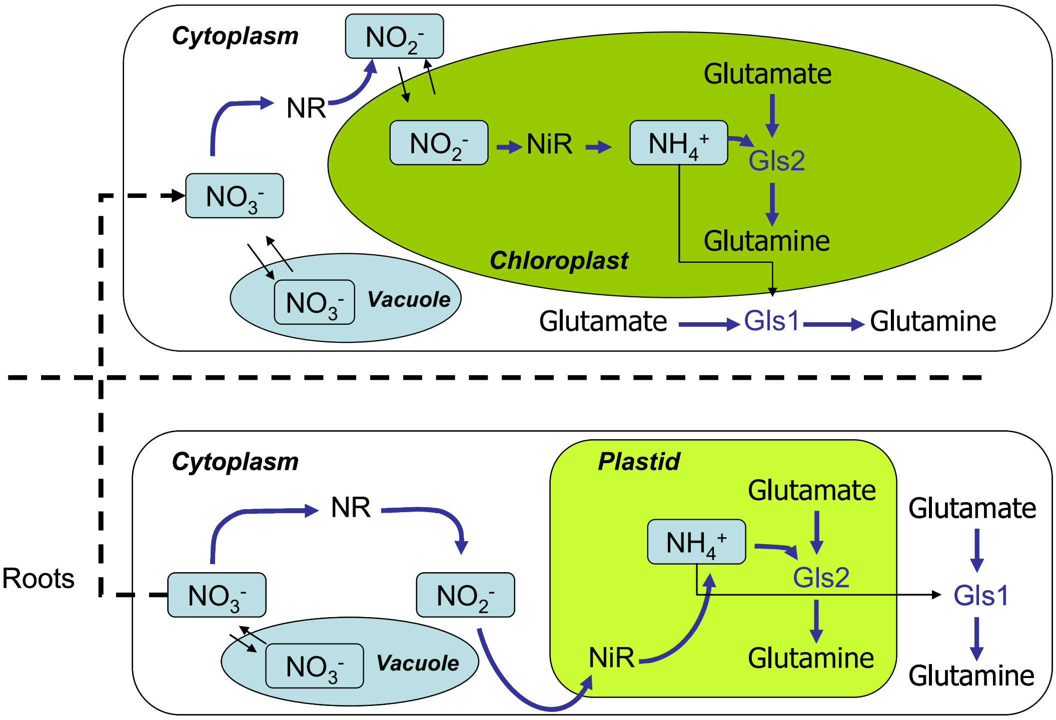

Fig. 1 Schematic and simplified representation of the nitrate uptake, transport and assimilation in plants. (Adapted from Crawford *et al.* 2000).

Table 1 Genes induced or inhibited in different plant organs exposed to low oxygen conditions.

Gene	Anoxic tissue	Induction/inhibition expression	Reference
GS1 (glutamine synthase)	Rice roots	Up-regulated	Reggiani *et al.* 2000
Nitrate reductase 1 (Nia 1)	Arabidopsis roots	Up-regulated	Klok *et al.* 2002
Nitrate reductase 2 (Nia 2)	Arabidopsis roots	Up-regulated	Klok *et al.* 2002
Class 1 haemoglobin	Arabidopsis roots	Up-regulated	Klok *et al.* 2002
Nitrate/chlorate transporter	Arabidopsis roots	Up-regulated	Klok *et al.* 2002
Glutamate decarboxylase	Arabidopsis roots	Up-regulated	Klok *et al.* 2002

crease NR activity and accelerate nitrate inclusion into amino acids, a phenomenon called "nitrate respiration". As shown for Arabidopsis root cultures, an oxygen deficit induces a two-stage response in which more than 210 genes (**Table 1**) are differentially expressed (Klok *et al.* 2002). Among those genes, both NR transcripts (*Nia 1* and *Nia2*) resulted significantly upregulated (**Table 1**). Although the upregulation of the NR activity has been largely described for anoxic tissues, recent papers speculate on the real importance of nitrate as a means to enhance plant resistance to anoxia (Libourel *et al.* 2006). Early experiments demonstrated that exogenous applications of nitrate are able to reduce acidosis in maize root tips with beneficial effects on anoxia tolerance (Fan *et al.* 1997, 1988). In order to study the fate of nitrate in anoxic plants, marked nitrate (^{15}N) was used to monitor the assimilation of nitrate during the anaerobic germination of rice (Raggiani *et al.* 1995; Reggiani and Bertani 2003). Results showed that ^{15}N was incorporated into amino acids, confirming that exogenous nitrate supplied during anoxia had been assimilated by plants. Moreover, an enhancement in nitrate utilisation can be considered valuable for the plant because its reduction in control conditions is proton consuming, but this option has been criticized because when glycolysis is the only source of reducing power, nitrate reduction generates protons (Libourel *et al.* 2006). Therefore, even if nitrate supply to flooded plants is known to increase the percentage of plant survival, the biochemical and molecular mechanisms underneath its positive effects are not fully understood.

A recent work suggested that nitrite, instead of nitrate, could play a pivotal role in mitigating cellular acidosis and contributing to plant resistance to oxygen deficiency (Libourel *et al.* 2006). It has been demonstrated that the exposure of anoxic maize roots to micromolar levels of nitrite is sufficient to confer the same effect of nitrate itself. Moreover, it has been observed that in anoxic roots the rate of

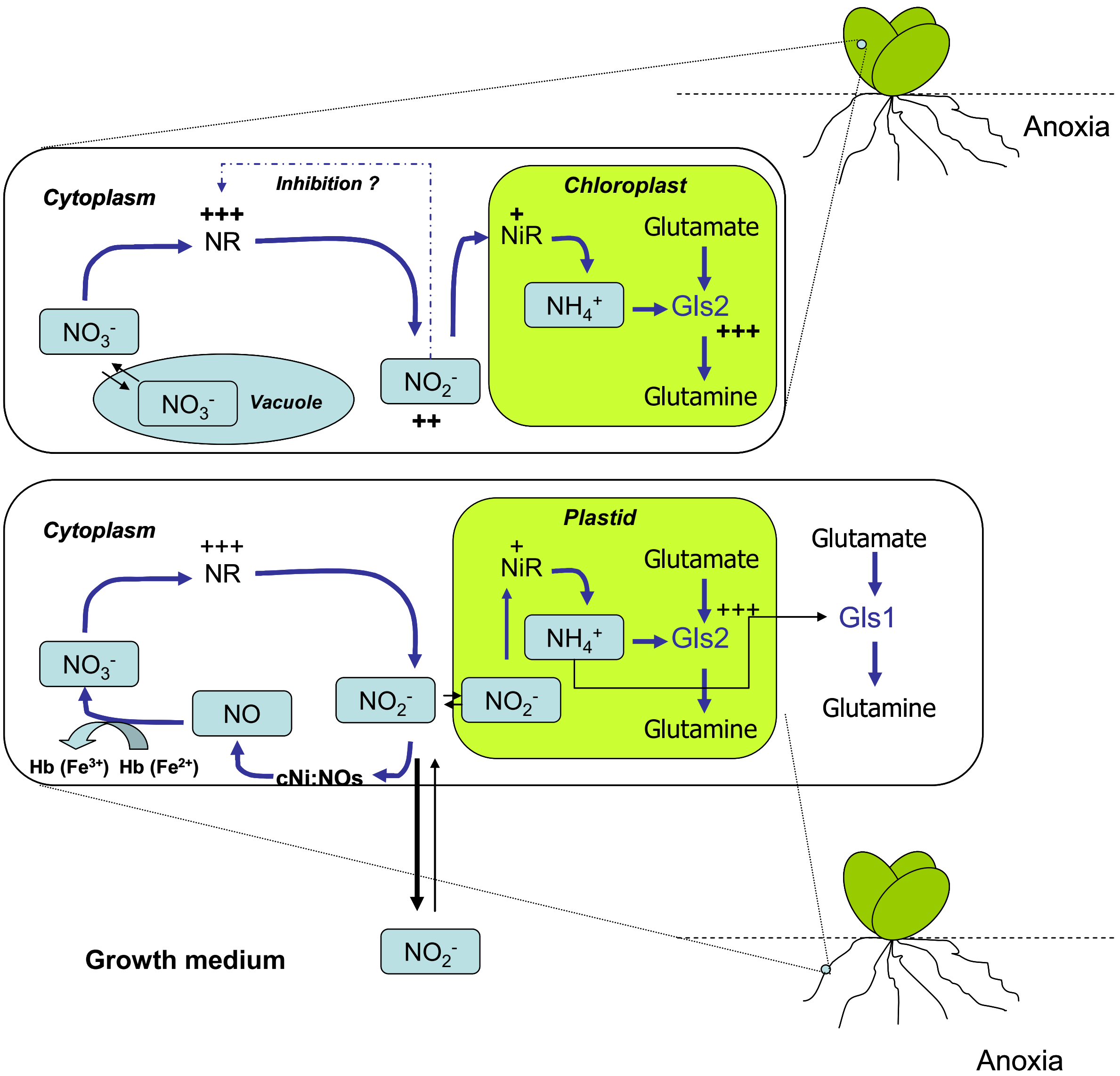

Fig. 2 Schematic and simplified representation of the nitrate uptake, transport and assimilation in plants grown under limited oxygen conditions.

nitrate reduction to ammonia is lower than in normoxic roots. This leads to an accumulation of nitrite, which is often secreted in the growing medium (Stoimenova *et al.* 2003; Allègre *et al.* 2004; Libourel *et al.* 2006). Similar results were obtained with tomato plants grown in hydroponics and exposed to long term anoxia: they displayed an increased nitrate uptake form the medium and an enhanced NR activity over a period of 55 h (Allègre *et al.* 2004). Nevertheless the root nitrites content decreased with the increasing amount of nitrites released in the nutrient solutions (Morard *et al.* 2004).

The data collected thus far show two peculiar modifications affecting the nitrate metabolic pathway and occurring during oxygen shortage: an upregulation in NR transcription and activity leading to the strong reduction of available nitrate ion to nitrite, and the consequent accumulation of nitrite that is scarcely used by the cell for protein synthesis and is eventually released in the external medium (**Fig. 2**). This accumulation suggests that downstream enzymes involved in nitrate assimilation could be repressed or shifted to other pathways. Analysis of the nitrate metabolism in anoxic spinach plants hydroponically grown suggests that the limiting step could be represented by NiR, even if the NiR gene was up-regulated in anoxic tissues (Antonacci and Ferrante 2007).

Aa accumulation

Anoxic treatments applied to root rice seedlings for 48 h induced an accumulation of amino acids with an increase of GS activity. Among the amino acids accumulated under anoxia, alanine and γ-Aminobutyric acid (GABA) represent the most abundant metabolites (Reggiani *et al.* 2000; Kato-Noguchi and Ohashi 2006).

NO and haemoglobin

Recently, it has been observed that roots of hypoxic or anoxic plants can produce NO in an oxygen-regulated way (Stöhr and Stremlau 2006). It has been also demonstrated that NO in plants is produced by NO synthases (NOS, E.C. 1.14.13.39) from L-arginine (Delledonne *et al.* 1998). Nitrites in limited oxygen conditions may be used for NO synthesis through plasma membrane-bound nitrite:NO reductase. A metabolic pathway that probably generates ATP and NAD^+ (Igamberdiev *et al.* 2005). Actually, some studies also confirm that NO can be produced by NR using nitrite and NADH (Yamasaki *et al.* 1999; Meyer *et al.* 2005; Stöhr and Stremlau 2006; Stoimenova *et al.* 2007). The first evidence of the NO production from nitrite came from soybean (Dean and Harper 1988). Functional studies in transgenic tobacco plants carrying antisense NiR gene showed an accumulation of nitrite with a high NO and N_2O production (Morot-Gaudry-Talarmain *et al.* 2002). The NO is a reactive free radical that may be toxic when highly produced in plant cells. It has been found that the NO is usually scavenged in nitrate through haemoglobin (Hb). Among the Hb isolated in plants, those that are expressed in hypoxic conditions belong to class-1 because they have high affinity for oxygen (Hunt *et al.* 2002). Functional analysis of Hb in plants has been performed using transgenic maize and *Arabidopsis* plants that over-expressed the Hb gene. Results showed that transformed plants were more tolerant to limited oxygen conditions (Hunt *et al.* 2002; Dordas *et al.* 2003).

Studies on the NO biosynthetic pathway conducted in *Nicotiana tabacum* L. roots and leaves exposed to hypoxia/anoxia demonstrated that nitrite is converted to NO using the mitochondrial electron chain only in roots. These results induced the authors to suggest that NO synthesis may require photosynthesis (Gupta *et al.* 2005).

Recent studies seem to confirm that the alkalinizing effect of cytoplasm is not due to nitrate but to nitrites which are converted to NO consuming H^+ (Libourel *et al.* 2006). Therefore, nitrites are essential for plant survival and nitrates are indirectly involved, since NR is also important for nitrites production and subsequently NO synthesis.

CONCLUSIONS

The main evidences of oxygen deficits on plant physiology are the drop in energy availability and pH acidification. Under these conditions, all ATP-demanding processes are slowed down or inhibited and protons accumulate in the cytoplasm. With respect to nitrate metabolism, a prolonged anoxic status may reduce nitrate uptake since its translocation inside the cell membrane is ATP-dependent and requires a $2H^+$ symport. Part of the radical nitrate pool may be subsequently reduced and transferred to shoots, as happens in normoxic conditions to many plant species. In the case of anoxic roots these events would cause nitrate depletion.

In contrast to these premises any publication regarding nitrate metabolism under low-oxygen conditions confirms the important connection existing between enzymatic pathways belonging to nitrate assimilation, along with intermediate and end-products, and the improved plant resistance to anaerobiosis. Several enzymes involved in nitrate metabolism have been proved to be responsive to oxygen deficits and so plural are the levels at which nitrate assimilation may work to sustain the cell metabolic status and improve plant resistance.

However, information about the role and regulation of some enzymes involved in nitrate metabolism in response to oxygen deficiency are still lacking. As the production of organic acids is essential for nitrate assimilation (Stitt 1999), the cross-talk between nitrogen and carbon metabolisms under oxygen deficits should be studied and the critical or limiting steps highlighted. Further research on the aerial part of the plant may provide new insights on the role of photosynthesis in plant stress adaptation upon progressive oxygen depletion.

Flooding and waterlogging are the major causes of low oxygen stress in plants. In the open field, oxygen deficiency is usually limited to the radical tissues, which become hypoxic and eventually anoxic, whereas the aerial part of the plant remains oxygenated. This is the main reason why most of the physiological, biochemical and molecular knowledge on plant response to oxygen deficits comes from experiments performed on radical tissues. Signalling molecules – nitric oxide being the most striking example – produced in anoxic tissues can diffuse and affect the entire plant physiology. On the other hand aerated shoots may contribute in alleviating hypoxic stresses and even increase plant tolerance by supplying oxygen to roots (Vartapetian *et al.* 2006). Some data on the effects of root anoxia on aerated shoots and *vice versa* are already available in the literature, but as many questions about root-shoot communication during hypoxia/anoxia still remain unanswered, the future research on plant stress response should be addressed to whole plant systems.

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